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13. ABSTRACT (*Maximum 200 words*)

We investigated the pharmacological classification of the subtypes of muscarinic receptors which are coupled to increased hydrolysis of phosphoinositides in rat cerebral cortex. Our results indicated that both M1 and M3 receptors mediate such a response. This response to muscarinic receptor stimulation is partially blocked by tetrodotoxin or by protein kinase C activators. The component of the response that is sensitive to blockade by such agents does not correspond to a certain receptor subtype. We investigated the role of protein kinase C in desensitization of muscarinic receptor function in a neuronal clone. While this kinase was clearly involved in the effects of phorbol esters, it did not play a role in agonist-induced receptor desensitization. We also studied some of the molecular events which accompany muscarinic receptor desensitization and down-regulation. These studies indicated that prolonged in vivo treatment of rats with an irreversible acetylcholinesterase decreased the concentration of the mRNA encoding the M2 muscarinic receptor.

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Regulation of Brain Muscarinic Receptors by Protein Kinase C

FINAL REPORT

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Statement of the Problem Studied:

The main purpose of the work was to elucidate the characteristics and the mechanisms of regulation of the signal transduction mechanisms coupled to neuronal muscarinic receptors, with emphasis on the role of protein kinase C.

Summary of Research Findings:

We have investigated the similarities in the mechanisms underlying the changes in muscarinic acetylcholine receptor sensitivity in neuronal tissue upon their exposure to protein kinase C-activating phorbol esters or to high concentrations of muscarinic agonists. Neuronal mouse neuroblastoma cells maintained in culture (clone N1E-115) were used as a model for these studies since they provide an excellent model to study regulation of the function of muscarinic receptors in the central nervous system.

Preincubation of cells with either phorbol esters of carbachol resulted in a significant loss of the ability of a subsequent addition of carbachol to stimulate cyclic GMP formation or phosphoinositide hydrolysis. However, it appears that different mechanisms mediate the effects of these two groups of agents on muscarinic receptor sensitivity. Thus, while the effects of phorbol esters are mediated by activation of protein kinase C, those of muscarinic agonists are independent of such a mechanism. Evidence for such divergent mechanisms is summarized as follows:

a. Desensitization induced by phorbol esters was heterologous while that induced by receptor agonists was homologous.

b. Only receptor desensitization induced by receptor agonists was accompanied by a significant decrease in the density of cell surface muscarinic receptors.

c. Effects of phorbol esters were reversed by protein kinase C inhibitors while those of agonists were not affected.

d. Depletion of cellular protein kinase C by prolonged incubation with phorbol esters significantly diminished the affects of these agents on muscarinic receptor sensitivity. However, this procedure had no effect on receptor desensitization induced by muscarinic agonists.

We have also studied the effects of phorbol esters and tetrodotoxin on muscarinic receptor-mediated phosphoinositide (PI) hydrolysis in rat cerebral cortex. Dissociated cerebral cortex cells were labeled with [^{3}H]inositol and labeled inositol phosphates were isolated by a chromatographic procedure. Muscarinic agonists increased PI hydrolysis by 3-4 fold in this

preparation. Phorbol esters caused only a partial (40-50%) inhibition of muscarinic receptor-mediated PI hydrolysis. Similar results were obtained using tetrodotoxin, a voltage-dependent sodium channel blocker. Neither of these two agents affected binding of ligands to muscarinic receptors. The partial inhibition of the PI response was not attributed to a selective blockade of the response mediated by a specific muscarinic receptor subtype.

In addition, we have investigated whether phosphoinositide (PI) hydrolysis in rat cerebral cortex is mediated through the activation of one or more muscarinic receptor subtypes. PI hydrolysis was measured in dissociated brain cell aggregates by a precursor labeling technique. Muscarinic agonist-induced activation of PI hydrolysis was antagonized in a concentration-dependent manner by several muscarinic receptor antagonists, including atropine, pirenzepine, telenzepine, AF-DX 116, 4-DAMP and HHSid. These inhibition curves were monophasic, except in the case of the m1 selective antagonists pirenzepine and telenzepine. In addition, Schild plots of pirenzepine were curvilinear. However, these curves were linearized after the selective alkylation of pirenzepine low affinity binding sites. These results suggest that at least two muscarinic receptor subtypes are coupled to activation of PI hydrolysis in rat cerebral cortex. The most likely candidates are the M1 and M3 receptor subtypes.

We have previously reported that the increase in guanylate cyclase activity by muscarinic receptor agonists in a neuronal cell line is closely related to the increase in phosphoinositide hydrolysis and mobilization of intracellular calcium ions. Based on these findings we have performed experiments which demonstrated that the role of this increased intracellular calcium is to trigger the formation of an intermediate derived from L-arginine, probably similar to endothelium-derived relaxing factor (EDRF) which would activate guanylate cyclase.

We continued investigating the mechanisms underlying agonist-induced regulation of muscarinic cholinergic receptors using Chinese hamster ovary cells stably transfected by the m1 muscarinic receptor gene. The m1 receptor sequence contains conserved aspartic acid residues in transmembrane regions II and III which are thought to contribute to the binding of the positively charges neurotransmitter acetylcholine. Interestingly, we discovered that mutation of the aspartic acid residue at position 71 to asparagine abolished agonist-induced down-regulation of receptor concentration.

Relationship between in vivo down-regulation of cardiac muscarinic receptors and changes in their encoding mRNA was investigated. Rats were treated either once or for ten days with the irreversible inhibitor of acetylcholinesterase, DFP, followed by measurements of cardiac acetylcholinesterase, in addition to the density and affinity of muscarinic receptors. Messenger RNA was

quantitated using the sensitive method of DNA-excess solution hybridization. Our data indicate that while short-term treatment resulted in a marked decrease in the density of cardiac muscarinic receptors, there was no accompanying significant change in the concentration of their mRNA. In contrast, long-term inhibition of acetylcholinesterase significantly decreased the concentration of mRNA coding for these receptors. These results are indicative of multiple mechanisms of down-regulation of cardiac muscarinic receptors, some of which might involve alterations at the transcriptional level.

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Report of inventions:

No inventions.